PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 30 August 2000 (30.08.00)	
International application No. PCT/GB99/04442	**Applicant's or agent's file reference** 44.69293/001
International filing date (day/month/year) 30 December 1999 (30.12.99)	**Priority date** (day/month/year) 05 January 1999 (05.01.99)
Applicant FRANTZEN, Frank	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 August 2000 (04.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
44.69293/001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/04442	30/12/1999	05/01/1999
Applicant		
AXIS-SHIELD ASA et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 4

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

[redacted]tional Application No
PCT/GB 99/04442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 726 322 A (AXIS BIOCHEMICALS AS) 14 August 1996 (1996-08-14) page 12, line 21 - line 40; claims 1-3	1-23
A	WO 93 15220 A (COCKBAIN JULIAN R M ;AXIS RESEARCH (NO)) 5 August 1993 (1993-08-05) page 14 page 24, paragraph 4 -page 25, paragraph 2	1-23

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 April 2000	26/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hart-Davis, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/04442

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0726322 A	14-08-1996	AT 142271 T	15-09-1996
		AU 676480 B	13-03-1997
		AU 4340893 A	01-09-1993
		BR 9305780 A	18-02-1997
		CA 2128512 A	05-08-1993
		CZ 9401763 A	15-12-1994
		DE 69304511 D	10-10-1996
		DE 69304511 T	23-01-1997
		DK 623174 T	13-01-1997
		EP 0623174 A	09-11-1994
		ES 2094524 T	16-01-1997
		FI 943462 A	14-09-1994
		WO 9315220 A	05-08-1993
		GR 3021365 T	31-01-1997
		HU 67550 A	28-04-1995
		JP 2870704 B	17-03-1999
		JP 8506478 T	16-07-1996
		NO 942729 A	15-09-1994
		SK 87894 A	12-04-1995
		US 5958717 A	28-09-1999
		US 5631127 A	20-05-1997
		US 5827645 A	27-10-1998
WO 9315220 A	05-08-1993	AT 142271 T	15-09-1996
		AU 676480 B	13-03-1997
		AU 4340893 A	01-09-1993
		BR 9305780 A	18-02-1997
		CA 2128512 A	05-08-1993
		CZ 9401763 A	15-12-1994
		DE 69304511 D	10-10-1996
		DE 69304511 T	23-01-1997
		DK 623174 T	13-01-1997
		EP 0623174 A	09-11-1994
		EP 0726322 A	14-08-1996
		ES 2094524 T	16-01-1997
		FI 943462 A	14-09-1994
		GR 3021365 T	31-01-1997
		HU 67550 A	28-04-1995
		JP 2870704 B	17-03-1999
		JP 8506478 T	16-07-1996
		NO 942729 A	15-09-1994
		SK 87894 A	12-04-1995
		US 5958717 A	28-09-1999
		US 5631127 A	20-05-1997
		US 5827645 A	27-10-1998

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
44.7.69293/001 jc		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/04442	30/12/1999	05/01/1999
International Patent Classification (IPC) or national classification and IPC G01N33/68		
Applicant AXIS-SHIELD ASA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/08/2000	19.03.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Montrone, M Telephone No. +49 89 2399 8711

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/04442

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1,3-19	as originally filed			
2,2a	as received on	23/02/2001	with letter of	22/02/2001

Claims, No.:

1-20	as received on	23/02/2001	with letter of	22/02/2001

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☒ the claims, Nos.: 21-23
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2,4,6,7,10,11,13-15,18,19
	No:	Claims	1,3,5,8,9,12,16,17,20
Inventive step (IS)	Yes:	Claims	
	No:	Claims	2,4,6,7,10,11,13-15,18,19
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents:

D1: EP-A-726322
D2: JP-A-4329357
D3: EP-A-483512
D4: US-A-4298592

The documents D2 to D4 were not cited in the international search report. Copies of the documents are appended hereto.

Item V:

The amendments to the claims and to the description, filed with the letter dated 22.02.01, have their basis in the originally filed application, and therefore satisfy Article 34(2) PCT.

1. Claim 1 refers to a method for assaying homocysteine wherein a sample is contacted with a SAH polyhapten, a SAH hydrolase and a primary antibody which can bind to said SAH polyhapten to produce a complex. Said primary antibody is furthermore capable to bind to adenosine or an adenosine analog as a co-substrate or to a conversion product of the SAH hydrolase. A complex will be formed which is indicative of the content of homocysteine in said sample. Said complex can be photometrically detected.

   The arguments of the applicant as brought forward in his letter of reply dated 22.02.2001 have been carefully considered. However, D1 is still considered to be detrimental to the novelty of the subject-matter of claims 1, 3, 5, 8, 9, 12, 16, 17 and 20 for the following reasons. Said document discloses several different methods for determining homocysteine. One of the photometric methods disclosed refers to particle agglutination and/or immunoprecipitation techniques by using polyclonal or monoclonal antibodies being directed against a polyhapten. SAH polyhapten is mentioned for raising specific antibodies. In addition, SAH-hydrolase is used (see page 6, line 37 to page line 24). The kit on page 12, lines 21 to 25 uses a furanose 6-thioester polyhapten instead of a SAH polyhapten since said furanose is

considered to be a "simplified" molecule in comparison to SAH (see page 7, line 36). However, D1 teaches only the use of the furanose polyhapten as a preferred simplified alternative towards the SAH polyhapten (see page 7, line 23 and 24). A preferred use does not exclude the use of SAH polyhapten, moreover, since antibodies were raised explicitly against SAH polyhapten which implies the use of said polyhapten in an assay consisting of an antibody/hapten complex formation. The inhibition of precipitation or particle agglutination occurs in the presence of S-adenosyl-homocysteine (SAH) thereby indicating that the antibodies used also react with a conversion product of the SAH-hydrolase. The photometric techniques used are turbidimetric and nephelometric. The haptens are conjugated to carrier molecules, such as BSA or haemocyanin.

D2 discloses a reagent wherein a chaotropic salt is added to a immunonephelometric assay containing PEG to additionally prevent unspecific interactions of antigen-antibody reactions (see abstract).

Consequently, the subject-matter of claims 1, 3, 5, 8, 9, 12, 16, 17 and 20 does not comply with the provisions of Article 33(2) PCT.

2. Moreover, the subject-matter of claims 2, 4, 6, 7, 10, 11, 13 to 15, 18 and 19 appears not to be inventive for the following reasons:

D1 is considered to be the closest prior art. Said document already discloses a method and a kit for the determination of homocysteine in an immunoassay format being measured by a turbidimetric or nephelometric procedure. The subject-matter of present claim 2 is distinguished therefrom by the addition of a secondary antibody. This addition results in an enhanced precipitation of the polyhapten:primary antibody complex.

The objective problem to be solved by the present application was therefore to further enhance the precipitation of a polyhapten:primary antibody complex.

However, the enhanced precipitation of antigen:antibody in the presence of a secondary antibody is known from D4 (see abstract, col. 1, line 57 to col. 2, line 35). Moreover, D4 discloses the advantageous effects of PEG with respect to accelerate

the immunoprecipitation, if PEG is additionally added to the reaction mixture (see col. 2, lines 36 to 68).

D3 discloses the accelerated immunoprecitpitation of antigen:primary antibody complexes in the presence of PEG, PVV or Dextran (see abstract, col. 1, lines 7 to 22, col. 2, lines 45 to 54, col. 3, lines 24 to 56). Moreover, a reagent is disclosed containing said non ionic polymers and an antibody (see col. 5, lines 41 to 55).

Thus, the person skilled in the art would have combined the teaching of D1 with D3 or D4 in order to solve the problem mentioned above and would have arrived at the claimed subject-matter falling within the scope of claim 2 without employing any inventive skill. Consequently, the subject-matter of present claim 2 does not appear to be inventive and does not fulfil the requirements of Article 33(3) PCT.

The subject-matter of claims 4, 6, 7, 10, 11, 13 to 15, 18 and 19 does not appear to add anything to the subject-matter of claim 1 which would render this claim inventive in the light of the cited documents. Thus, said claims do not fulfil the requirements of Article 33(3) PCT either.

Item VIII:

The term "hapten moieties the hapten" used in claim 1, line 5 is unclear and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT).

Q2

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF RECEIPT OF SEARCH COPY

(PCT Rule 25.1)

To:
FRANK B. DEHN & CO.
Attn. COCKBAIN, Julian
179 Queen Victoria Street
London EC4V 4EL
UNITED KINGDOM

Date of mailing *(day/month/year)*	31/01/2000
Applicant's or agent's file reference	IMPORTANT NOTIFICATION
44.69293/001	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB 99/04442	30/12/1999	05/01/1999

Applicant

AXIS-SHIELD ASA et al.

1. **Where the International Searching Authority and the Receiving Office are not the same office:**

The applicant is hereby notified that the search copy of the international application was received by this International Searching Authority on the date indicated below.

Where the International Searching Authority and the Receiving Office are the same office:

The applicant is hereby notified that the search copy of the international application was received on the date indicated below.

19/01/2000 *(date of receipt).*

2. ☐ The search copy was accompanied by a nucleotide and/or amino acid sequence listing in computer readable form.

3. **Time limit for establishment of International Search Report**

The applicant is informed that the time limit for establishing the International Search Report is 3 months from the date of receipt indicated above or 9 months from the priority date, whichever time limit expires later

4. A copy of this notification has been sent to the International Bureau and, where the first sentence of paragraph 1 applies, to the Receiving Office.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016	ISA/EP

Form PCT/ISA/202 (July 1998)

****** COPY FOR RECEIVING OFFICE ******

SP

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION (PCT Rule 44.1)

To:
FRANK B. DEHN & CO.
Attn. COCKBAIN, Julian
179 Queen Victoria Street
London EC4V 4EL
UNITED KINGDOM

FILE 69 293/ 00/
28 APR 2000
RECEIVED
ANSD

Date of mailing (day/month/year)	26/04/2000
Applicant's or agent's file reference: 44.69293/001	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No.: PCT/GB 99/04442	International filing date (day/month/year): 30/12/1999
Applicant: AXIS-SHIELD ASA et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41–22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

DUE DATES NOTED

Name and mailing address of the International Searching Authority: European Patent Office, P.B. 5818 Patentlaan 2, NL-2280 HV Rijswijk, Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer: Jaap Hurenkamp

26/6/00

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
44.69293/001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/04442	30/12/1999	05/01/1999
Applicant		
AXIS-SHIELD ASA et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 4

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/04442

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0726322 A	14-08-1996	AT 142271 T	15-09-1996
		AU 676480 B	13-03-1997
		AU 4340893 A	01-09-1993
		BR 9305780 A	18-02-1997
		CA 2128512 A	05-08-1993
		CZ 9401763 A	15-12-1994
		DE 69304511 D	10-10-1996
		DE 69304511 T	23-01-1997
		DK 623174 T	13-01-1997
		EP 0623174 A	09-11-1994
		ES 2094524 T	16-01-1997
		FI 943462 A	14-09-1994
		WO 9315220 A	05-08-1993
		GR 3021365 T	31-01-1997
		HU 67550 A	28-04-1995
		JP 2870704 B	17-03-1999
		JP 8506478 T	16-07-1996
		NO 942729 A	15-09-1994
		SK 87894 A	12-04-1995
		US 5958717 A	28-09-1999
		US 5631127 A	20-05-1997
		US 5827645 A	27-10-1998
WO 9315220 A	05-08-1993	AT 142271 T	15-09-1996
		AU 676480 B	13-03-1997
		AU 4340893 A	01-09-1993
		BR 9305780 A	18-02-1997
		CA 2128512 A	05-08-1993
		CZ 9401763 A	15-12-1994
		DE 69304511 D	10-10-1996
		DE 69304511 T	23-01-1997
		DK 623174 T	13-01-1997
		EP 0623174 A	09-11-1994
		EP 0726322 A	14-08-1996
		ES 2094524 T	16-01-1997
		FI 943462 A	14-09-1994
		GR 3021365 T	31-01-1997
		HU 67550 A	28-04-1995
		JP 2870704 B	17-03-1999
		JP 8506478 T	16-07-1996
		NO 942729 A	15-09-1994
		SK 87894 A	12-04-1995
		US 5958717 A	28-09-1999
		US 5631127 A	20-05-1997
		US 5827645 A	27-10-1998

Claims:

1. A method for assaying homocysteine in a sample, said method comprising:

contacting said sample with two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a second antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes whereby the quantity of said complex produced is indicative of the content of homocysteine in said sample; and photometrically detecting said complex.

2. A method as claimed in claim 1 wherein at least one of said reagents contains a said secondary antibody.

3. A method as claimed in claims 1 or 2 wherein said complex is determined nephelometrically or turbidimetrically.

4. A method as claimed in any of claims 1 to 3 wherein photometric determinatination takes place before complex generation is complete.

5. A method as claimed in any of claims 1 to 4 wherein said sample is a a serum or plasma sample.

6. A method as claimed in any of claims 1 to 5 wherein at least one of said reagents additionally

contains an agent which promotes precipitation of said complex.

7. A method as claimed in claim 6 wherein said agent which promotes precipitation is polyethylene glycol.

8. A method as claimed in any of claims 1 to 7 wherein at least one of said reagents further comprises a carrier protein.

9. A method as claimed in any of claims 1 to 8 wherein said polyhapten consists of a backbone structure onto which the haptens are bound.

10. A method as claimed in claim 9 wherein said backbone structure is porcine thyroglobulin.

11. A method as claimed in any of claims 1 to 10 wherein the hapten of said polyhapten is S-adenosine homocysteine (SAH).

12. A method as claimed in any of claims 1 to 11, wherein at least one of said reagents contains said primary and secondary antibodies and additionally contains a chaotropic salt.

13. A homocysteine assay reagent kit comprising two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a second antibody capable of binding to said complex, said primary

antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes.

14. A kit as claimed in claim 13 wherein at least one of said reagents contains a said secondary antibody.

15. A kit as claimed in either of claims 13 or 14, wherein at least one of said reagents additionally contains an agent which promotes precipitation of said complex.

16. A kit as claimed in claim 15 wherein said agent which promotes precipitation is polyethylene glycol.

17. A kit as claimed in any of claims 13 to 16 wherein at least one of said reagents further comprises a carrier protein.

18. A kit as claimed in any of claims 13 to 17 wherein said polyhapten consists of a backbone structure onto which the haptens are bound.

19. A kit as claimed in claim 18, wherein said backbone structure is porcine thyroglobulin.

20. A kit as claimed in any of claims 13 to 19, wherein the hapten of said polyhapten is S-adenosine homocysteine (SAH).

21. A kit as claimed in any of claims 13 to 20 wherein at least one of said reagents contains said primary and secondary antibodies and additionally contains a chaotropic salt.

22. A kit as claimed in any of claims 13 to 21

containing two said reagents.

23. An aqueous assay reagent containing a chaotropic salt, an immuno-precipitation enhancer, a primary antibody and a secondary antibody.

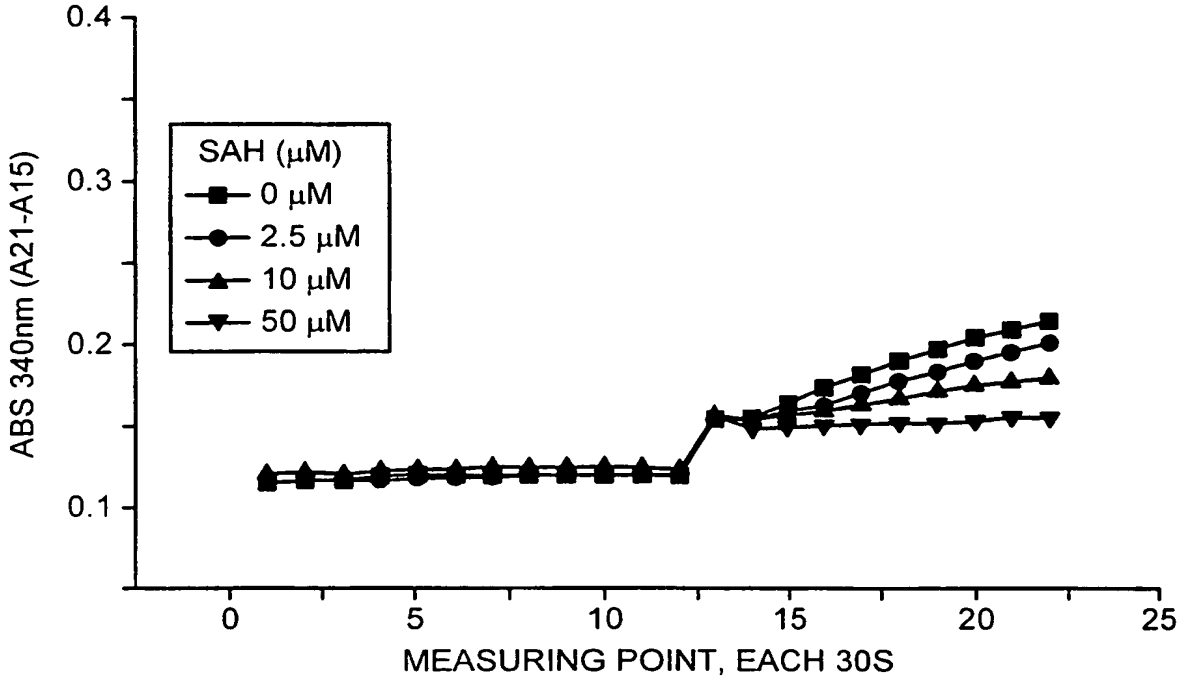

0.4
0.3
0.2
0.1
ABS 340nm (A21-A15)
SAH (μM)
0 μM
2.5 μM
10 μM
50 μM
0
5
10
15
20
25
MEASURING POINT, EACH 30S

FIG. 1

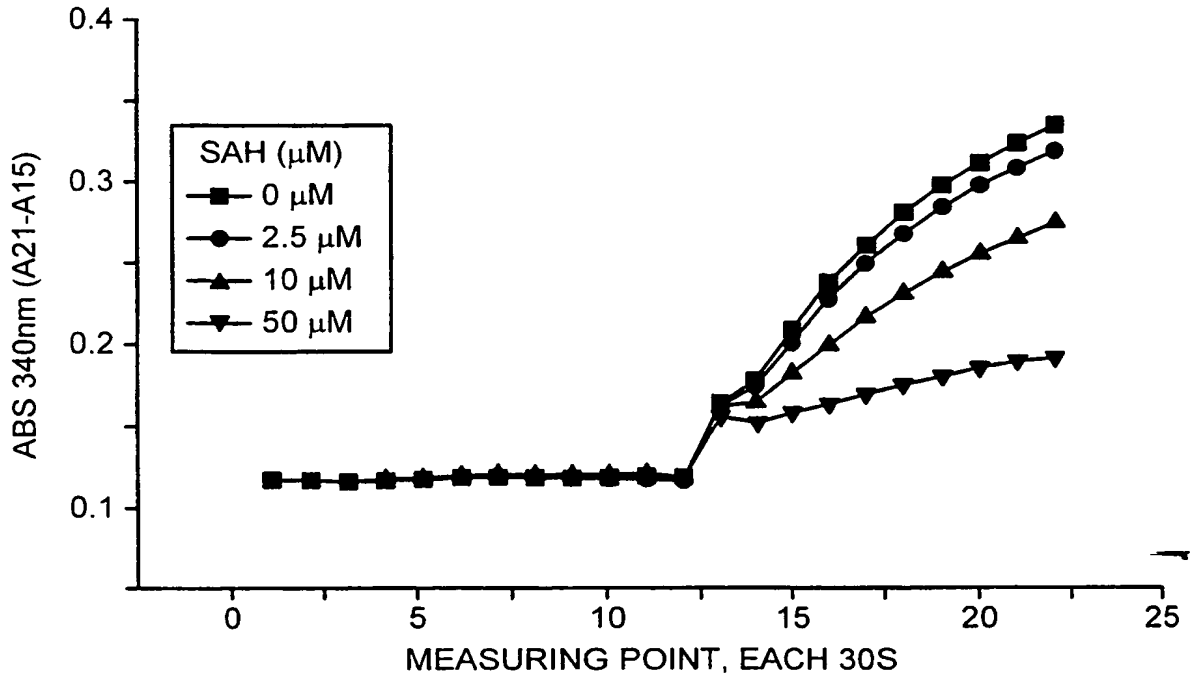

0.4
0.3
0.2
0.1
ABS 340nm (A21-A15)
SAH (μM)
0 μM
2.5 μM
10 μM
50 μM
0
5
10
15
20
25
MEASURING POINT, EACH 30S

FIG. 2

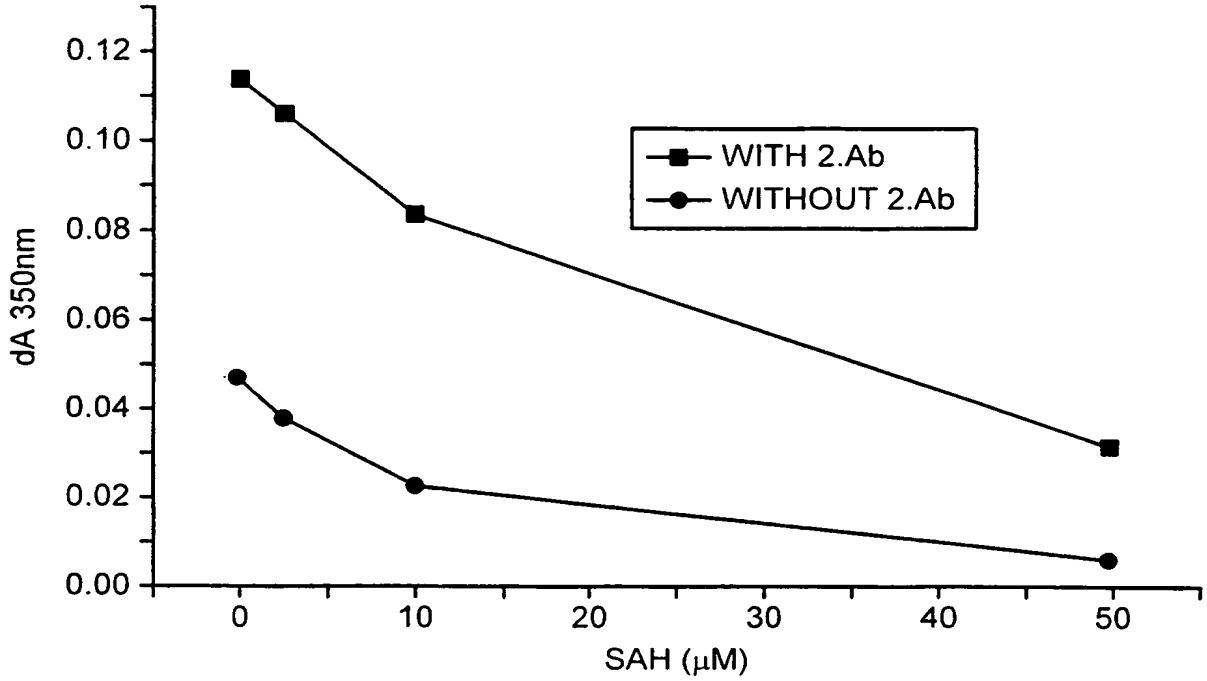

0.12
0.10
0.08
0.06
0.04
0.02
0.00
dA 350nm
WITH 2.Ab
WITHOUT 2.Ab
0
10
20
30
40
50
SAH (µM)

FIG. 3

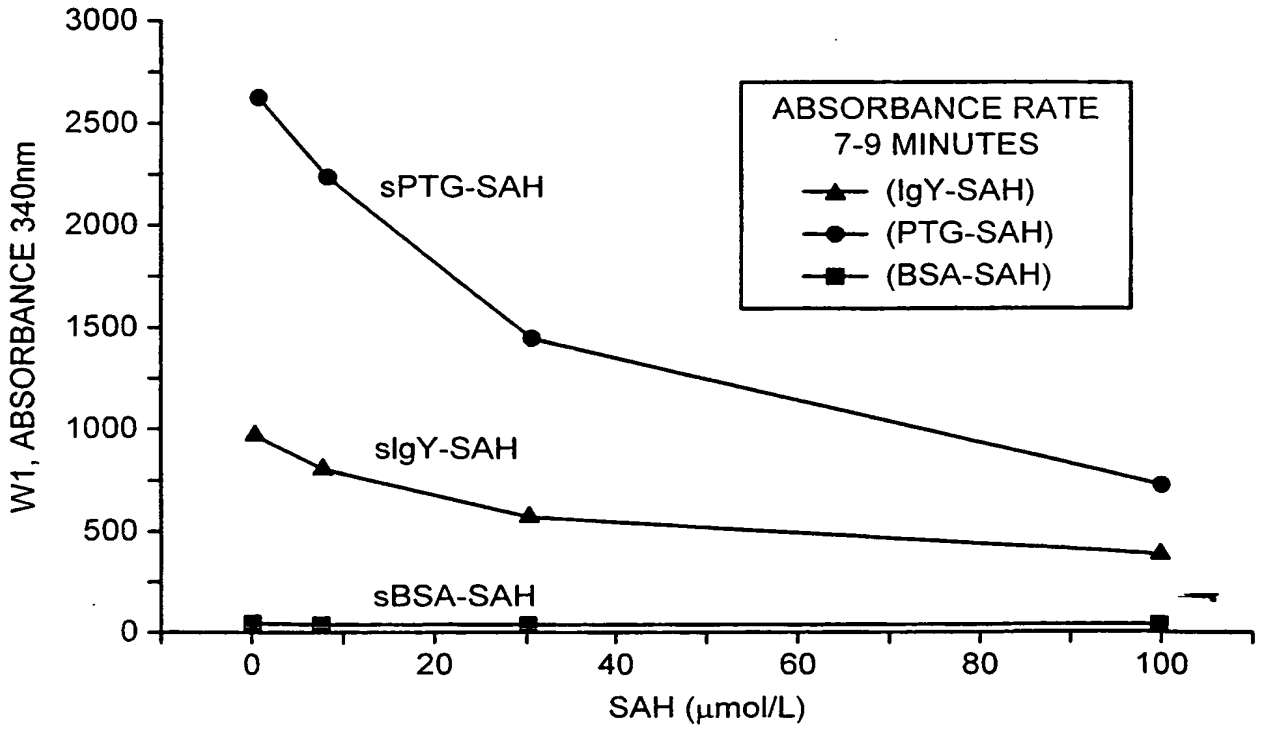

3000
2500
2000
1500
1000
500
0
W1, ABSORBANCE 340nm
ABSORBANCE RATE
7-9 MINUTES
(IgY-SAH)
(PTG-SAH)
(BSA-SAH)
sPTG-SAH
sIgY-SAH
sBSA-SAH
0
20
40
60
80
100
SAH (µmol/L)

FIG. 4

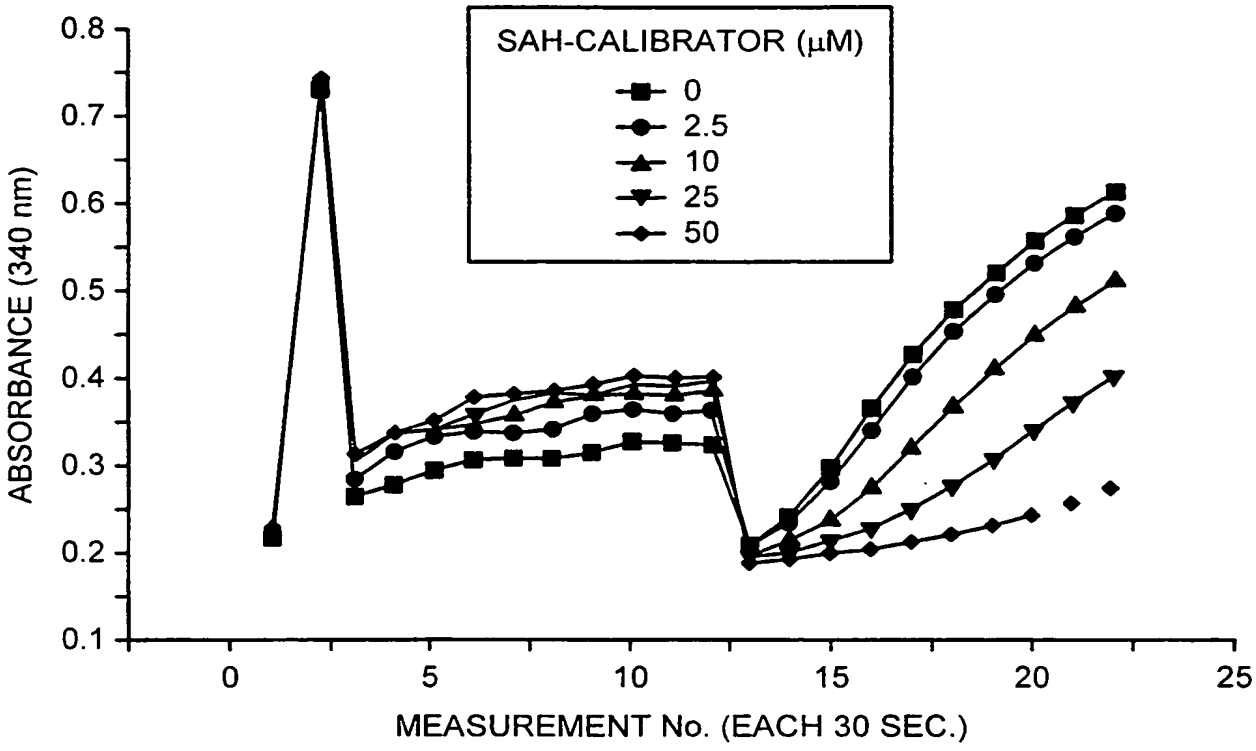

SAH-CALIBRATOR (μM)
0
2.5
10
25
50
ABSORBANCE (340 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
MEASUREMENT No. (EACH 30 SEC.)

FIG. 5

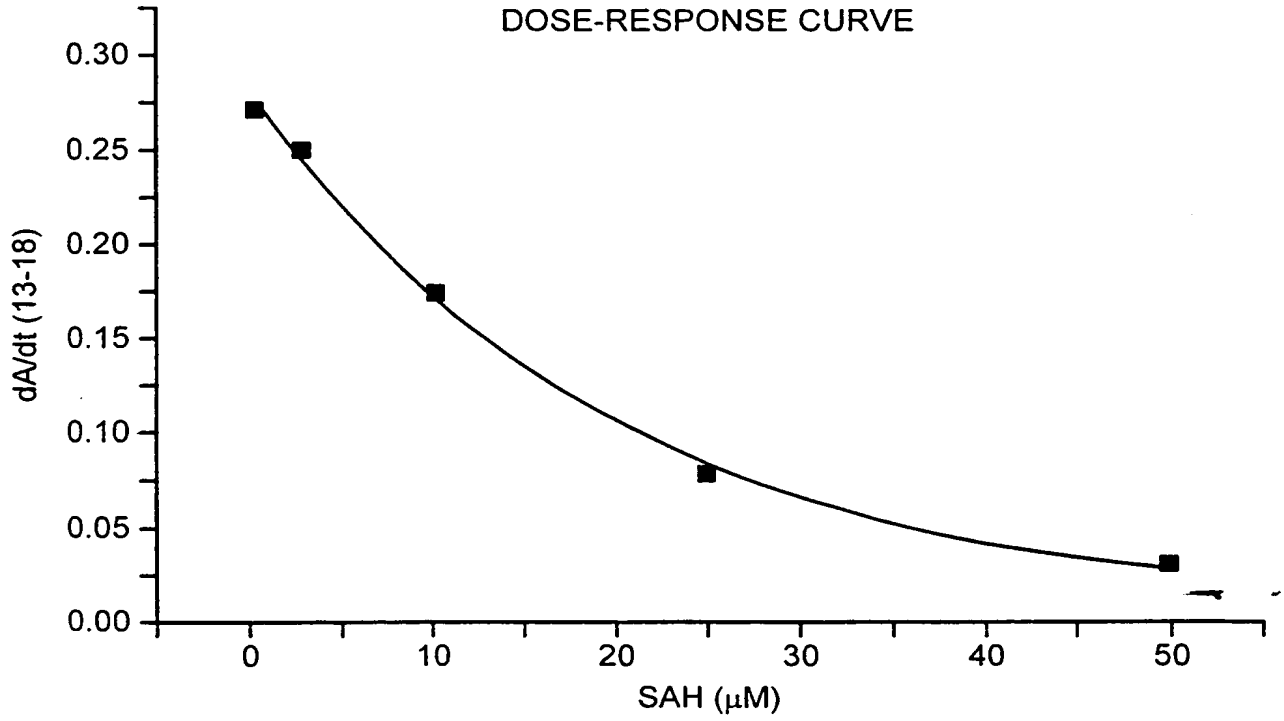

DOSE-RESPONSE CURVE
dA/dt (13-18)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
SAH (μM)

FIG. 6

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/68

A1

(11) International Publication Number: WO 00/40973

(43) International Publication Date: 13 July 2000 (13.07.00)

(21) International Application Number: PCT/GB99/04442

(22) International Filing Date: 30 December 1999 (30.12.99)

(30) Priority Data:
9900159.6 5 January 1999 (05.01.99) GB

(71) Applicant *(for all designated States except US)*: AXIS–SHIELD ASA [NO/NO]; Ulvenveien 87, P.O. Box 206 Økern, N–0510 Oslo (NO).

(71) Applicant *(for GB only)*: COCKBAIN, Julian [GB/GB]; Flat 4, 83 Linden Gardens, London W2 4EU (GB).

(72) Inventor; and

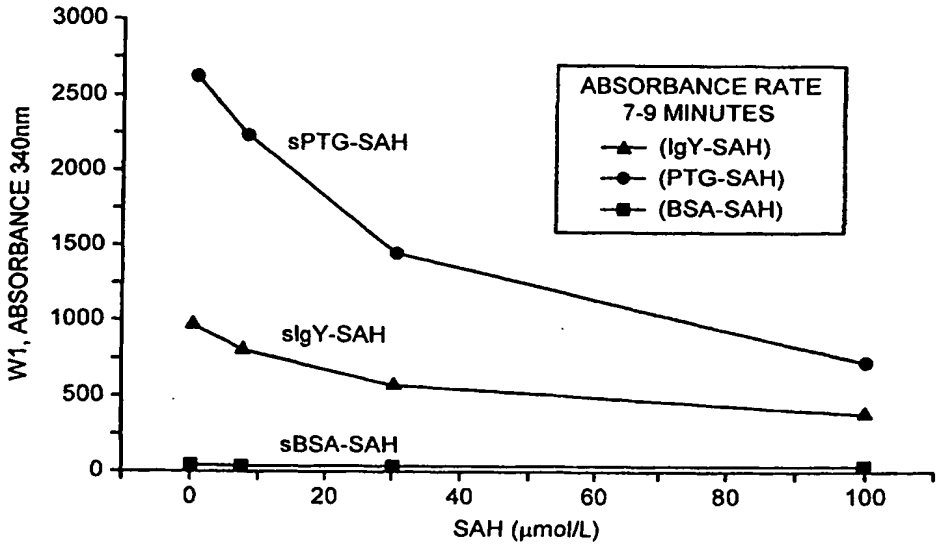
(75) Inventor/Applicant *(for US only)*: FRANTZEN, Frank [NO/NO]; Axis–Shield ASA, Ingeniørgården, N–8031 Bodø (NO).

(74) Agents: COCKBAIN, Julian et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ASSAY FOR HOMOCYSTEINE



(57) Abstract

The invention provides a method for assaying homocysteine in a sample, said method comprising: contacting said sample with two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co–substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co–substrate or a conversion product of said homocysteine converting enzyme, and a second antibody capable of binding to said complex, said primary antibody also being capable of binding to said co–substrate or a conversion product of one of said enzymes whereby the quantity of said complex produced is indicative of the content of homocysteine in said sample; and photometrically detecting said complex.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Assay for Homocysteine

The invention relates to an assay for homocysteine in body fluids or fluids derived therefrom and to kits for such assays.

Homocysteine is a sulphur-containing amino acid that is closely related to methionine and cysteine. There is no DNA-coding for homocysteine and it is not present in naturally occurring proteins. Homocysteine is formed in the metabolism of the essential amino acid methionine. In plasma about 1% of homocysteine exists in the free reduced form, about 70% is bound to albumin and the rest is present in low molecular weight disulphides, predominantly with cysteine. Total homocysteine, a term describing all molecular forms of homocysteine (e.g. free and bound) is abbreviated hereinafter as tHcy. In the literature the term homocysteine is often abbreviated as Hcy and used to describe both free and bound homocysteine.

Hyperhomocysteinemia is generally defined as fasting plasma tHcy above 15 μmol/L. It has been shown to be a risk factor for cardiovascular disease and for complications in pregnancy and congenital malformations. A link between impaired homocysteine metabolism and neuropsychiatric disorders and cognitive impairment in the elderly has also been shown.

Normalisation of homocysteine levels may be achieved by life-style changes (e.g. quitting smoking, taking exercise, reducing coffee consumption, improving diet, etc.) or by vitamin supplementation.

Accordingly there has been significant emphasis in recent years on the development of assays for homocysteine. Such assays are available for example from Abbott Laboratories (US), Axis-Shield (UK and NO), Bio-Rad (NO), and A/C Diagnostics (US). Such assays are moreover described in patent applications from Axis-

Shield (NO) and the University of Glasgow (UK), e.g. US-A-5631127 and WO98/07872.

Direct determination of homocysteine is not straightforward and these assays generally involve enzymatic conversion of homocysteine and direct or indirect determination of a homocysteine conversion product. Thus for example US-A-5631127 describes conversion of homocysteine and adenosine to S-adenosyl homocysteine (SAH) using SAH-hydrolase and determination of SAH, while WO98/07272 describes the conversion of homocysteine and water to α-ketobutyrate and hydrogen sulphide using homocysteine desulphurase and determination of either the α-ketobutyrate or the hydrogen sulphide.

For homocysteine to be available as an analytical parameter for most clinical laboratories however, a homocysteine assay is needed which may be performed in the commercially available automated clinical chemistry analysers, sometimes referred to as clinical chemistry platforms, for example the systems available from Hitachi and Roche Diagnostics.

Generally clinical chemistry analysers are characterised by the use of simple photometric measurements, e.g. colorimetric, turbidimetric or nephelometric measurements. Additionally the number of manual handling steps for running assays on these analysers have to be as few as possible. Preferably little or no sample pre-treatment should be required and ideally the reagents used should be assay-ready in a stable form. The number of compartments for reagents in these analysers is typically extremely limited, and since the analysers may be arranged to determine several different analytes in a sample, often only two or three reagent compartments may be available for an assay for a single analyte.

Furthermore, the total assay time required per sample must be relatively short both to optimise

throughput and to avoid disturbing the instrument logistics and hence the instrument's handling of different analytes.

The existing homocysteine assays mentioned above are not generally applicable to a range of automated analysers, in particular since the reagents are not sufficiently few in number and in ready-to-use stable form. Thus for example the Enzymatic Homocysteine Assay Kit from A/C Diagnostics has three buffer solutions and four reagents which have to be dissolved in the buffers before use. In the Abbott IMx system, four reagents are required - these are all kept in separate compartments and cannot, due to stability and incompatibility problems, be mixed or combined to reduce the total number of reagents. The Shield Homocysteine Chromogenic Assay likewise uses four reagents.

We have now found that, using a polyhapten, a homocysteine converting enzyme and an antibody, it is possible to produce an immunoassay for homocysteine which requires only two, or at most three, stable, ready-to-use reagents.

Viewed from one aspect therefore the invention provides a method for assaying homocysteine in a sample, said method comprising:

contacting said sample with two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a secondary antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes whereby the quantity of said complex produced is indicative of the content of

homocysteine in said sample; and photometrically detecting said complex.

The assay of the invention may be used to determine tHcy or free homocysteine; in the former and more usual case the sample will generally be treated with a reducing agent to liberate bound homocysteine.

By "stable" it is meant that the aqueous reagents should be capable after storage at 20°C for at least 3 months, preferably at least 6 months, more preferably at least 18 months, of being used to determine homocysteine content in a 10 μmol/L L-homocysteine standard with a loss in accuracy of no more than 10% preferably no more than 5% as compared with freshly prepared equivalent reagents. In testing stability, determination of the antibody:polyhapten complex may be performed turbidimetrically using a Hitachi 911 apparatus.

Viewed from a further aspect the invention also provides a homocysteine assay reagent kit comprising two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a secondary antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes.

In the method of the invention, the homocysteine content of the sample, preferably the tHcy value, is determined indirectly by determining the amount of the polyhapten:antibody complex, preferably by nephelometry or turbidimetry. The homocysteine content may be determined quantitatively, e.g. in absolute units such as μmol/L, or alternatively the determination may be

qualitative, e.g. simply that it is above a predetermined threshold such as 15, 18 or 20 μmol/L. Generally, the assay measurement will be calibrated against standard homocysteine solutions containing known concentrations of homocysteine, usually L-homocysteine; however for assays run on automated analysers only occasional calibration will be necessary, e.g. when reagent reservoirs are refilled.

Where an antibody is used in the assay method and kits of the invention, this may be a polyclonal or more preferably a monoclonal antibody; however the term antibody is also used to cover single chain antibodies, antibody fragments (e.g. Fab fragments), and oligopeptides and oligonucleotides capable of highly specific binding to the polyhapten or polyhapten:primary antibody complex. Appropriate such specific binding oligonucleotide and oligopeptides may be identified by conventional combinatorial chemistry techniques, e.g. using phage display libraries. Antibodies to the hapten in the polyhapten, ie. to the direct or indirect homocysteine conversion product of the homocysteine conversion enzyme, the co-substrate, or to the polyhapten: primary antibody complex may also be raised in a conventional fashion, e.g. by mouse immunization followed by fusion with myeloma cells, etc.

In the assay method of the invention, the aqueous reagents are preferably contacted with the sample sequentially with the analyte determination taking place after addition of the final reagent. However, unlike earlier assays, if the time of determination is carefully controlled, determination may take place before complex generation is complete: in this way the time required for performing the assay may be reduced, e.g. to less than 15 minutes, advantageously less than 10 minutes and especially advantageously less than 5 minutes.

The sample investigated using the method of the

invention may be any homocysteine containing sample. Generally it will be a body fluid or a liquid derived from a body fluid, e.g. blood, urine, cerebrospinal fluid, serum or plasma. Serum is preferred and plasma is particularly preferred. Where the sample is one in which homocysteine is protein bound (e.g. blood, serum, plasma, etc.) it is preferred that one of the reagents, preferably the first reagent, added to the sample, should contain a reducing agent so as to liberate the free reduced homocysteine. Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are preferred as reductants. TCEP is especially preferred since it is a strong reductant which is stable at pH values of about 7. Other pH 7 stable reductants could however be used if desired.

The first reagent also preferably contains the co-substrate for the homocysteine converting enzyme, if such a co-substrate is required. Thus for example where the homocysteine converting enzyme is SAH-hydrolase it is desirable to include adenosine or an adenosine analog in the reagents.

In general one or more of the reagents will preferably contain an agent which promotes precipitation of the polyhapten:primary antibody complex. Suitable such materials include polysaccharides, polyhydroxyl compounds, carbohydrates, alginates, chitosan, heparin, heparinoids, poloxamers and poloxamines. Especially preferred are polyalkylene oxides such as polethylene glycol (PEG). PEG of molecular weight 1 to 10 kD, particularly 2 to 8 kD and more particularly about 6 kD, functions particularly well in this regard.

Besides agents such as PEG, complex precipitation may be enhanced by reducing pH to acid levels. This however is less preferred.

Less preferably, immunocomplex precipitation may be enhanced using organic solvents or salts, e.g. ammonium acetate, metal cations, 2-ethyl-6,9-diaminoacridine

lactate, protamine, polyacrylates, etc.

Since agents which promote complex precipitation may also promote precipitation of other proteins present in the sample, where such agents are used they are preferably present in two or all three of the reagents.

Depending on whether two or three reagents are used according to the invention, the different components may be distributed differently in the reagents. For a two reagent system, the first reagent desirably contains an immunocomplex precipitation promoter (e.g. PEG), a co-substrate (e.g. adenosine) if such is necessary, a reductant (e.g. TCEP), and a polyhapten (e.g. poly-SAH), while the second reagent desirably contains the primary antibody (e.g. anti-SAH), the homocysteine converting enzyme (e.g. SAH-hydrolase), a complex precipitation promoter (e.g. PEG) and, if desired, the secondary antibody. Where three reagents are used, two or three preferably contain an immunocomplex precipitation promoter (e.g. PEG); while the primary antibody and the homocysteine converting enzyme are preferably not formulated together with the polyhapten or, where present, the co-substrate.

If desired the reagents used may include a carrier protein, e.g. casein or albumin, both as a stabilizer and to compensate for variations in the background signal from proteins in the sample (e.g. plasma proteins) which may occur at varying levels in different samples - by adding carrier protein the percentage variation between samples in the proteins responsible for background signal is reduced.

In the assay method of the invention, an analyte competes with the polyhapten for binding to the primary antibody. The analyte may be a homocysteine co-substrate or a homocysteine conversion product or (where a second enzyme is used) a conversion product of any of these. The polyhapten preferably is a water-soluble compound or compound mixture which has at least two,

preferably at least 5, more preferably at least 10 and especially preferably at least 50 hapten moieties which are the same as or similar to at least part of the analyte such that the primary antibody is capable of binding both to the analyte and the hapten moieties of the polyhapten. More generally, the polyhapten preferably has at least one hapten moiety per 100 kD of its molecular weight and the polyhapten preferably has a molecular weight of at least 500 kD, more preferably at least 1 MD, e.g. up to 3 MD. Conveniently, it has a polymeric backbone structure onto which the haptens are bound, e.g. a polypeptide structure (for example a protein such as a thyroglobulin or a polypeptide such as polyaspartic acid), a polysaccharide (such as a dextran derivative, alginic acid and mannan), or a synthetic polymer with pendant functional groups such as hydroxyl, carboxyl and/or amino groups. Porcine thyroglobulin, which has a molecular weight of 660 kD, is especially preferred since its size is appropriate and since it can be dimerized to produce a larger polyhapten. In general proteins and polypeptides will be preferred as polyhapten backbone materials since their isoelectric points, and hence the precipitation characteristics of the polyhapten:primary antibody complex, can readily be modified. The hapten moieties may be coupled to the polyhapten backbone using conventional chemistry, e.g. using peptide coupling between amine and carboxyl groups of the hapten and the polyhapten-backbone compound.

Thus, by way of example, SAH can be coupled to porcine thyroglobulin using EDC/EDAC (1-ethyl-3-(dimethylaminopropyl)-carbodiimide) or another carboxyl activator, optionally after treating the protein with succinic acid. A one-pot reaction is possible, but it is preferred to succinylate the protein in a separate step before conjugation to SAH. Succinic acid couples to free amino groups on the protein, provides a pendant carboxyl for SAH attachment and also lowers the

isoelectric point of the protein. The carboxyl activator permits coupling of SAH carboxyls with protein amino groups as well as of SAH amino groups with native and succinic acid-deriving carboxyls on the proteins. In such a reaction, N-hydroxy succinimide may also be used to promote SAH:protein coupling.

As mentioned above, the polyhapten is preferably water-soluble. However it is also possible to use colloidal particles, for example nano-particles, e.g. particles having sizes in the 1 to 1000 nm, especially 50 to 800 nm range, as a matrix onto which the haptens are bound. The particle size should be such that the particles form a stable aqueous dispersion from which they do not settle out. The particles may be of a synthetic polymer with suitable functional groups for hapten attachment.

The homocysteine converting enzyme used in the assay method may be any enzyme capable of directly or indirectly using or generating an antigenic analyte. Typical enzymes include SAH-hydrolase, homocysteinase (homocysteine desulphurase), methionine synthase, cystathionine β-synthase, and betaine-homocysteine methyl transferase. Where the enzyme used is SAH-hydrolase, the analyte will generally be adenosine or SAH or an enzymic conversion product of one of these; however SAH is the preferred analyte. Where the enzyme used is homocysteine desulphurase, the analyte will generally be an enzyme conversion product of α-ketobutyrate.

In the assay method of the invention, a secondary antibody which binds to the polyhapten:primary antibody complex may be used if desired. In this way precipitation of the polyhapten:primary antibody complex is enhanced and the sensitivity of the assay may be improved. The secondary antibody may for example be an anti-mouse IgG polyclonal antibody where the primary antibody is a mouse antibody. The secondary antibody

may be polyclonal or monoclonal. The secondary antibody can be of any origin, e.g. rabbit, chicken, etc., as long as its cross-reactivity with components in the sample under investigation is not such as to cause any change in light transmission or scattering. Thus cross-reactivity with human plasma proteins and antibodies is desirably absent.

The secondary antibody need not be a traditional antibody: any specific binding material which enhances precipitation of the polyhapten:primary antibody complex may be used, e.g. antibody fragments, oligopeptides, oligonucleotides, single chain antibodies, etc., optionally with two or more coupled together.

The use of secondary antibodies however potentially increases the compatibility problems, ie. the problems faced in producing an assay method requiring only two or three reagents. For a three reagent system, it is possible to have primary and secondary antibodies and polyhapten in separate reagents. However, it is possible to formulate the primary and secondary antibodies together if a chaotropic salt is also included in the reagent. Such chaotropic salts include for example salts containing SCN^-, CCl_3COO^-, CF_3COO^-, Cs^+, Li^+, or Mg^{2+} ions and guanidine chloride.

The twin antibody reagent will preferably also contain a reagent which promotes precipitation of the immunocomplex, e.g. a polyalkylene oxide such as PEG or other such compounds as discussed earlier. Thus the antibodies, PEG and chaotropic salt will be at a higher concentration than in the reaction medium when they are mixed with sample and polyhapten. By combining a small volume of this antibody containing reagent with a larger volume of another solution, e.g. another reagent mixed with the sample, dilution of the chaotropic salt will permit immunoprecipitation to begin. This novel strategy can be used in other assays to obtain signal enhancement and forms a further aspect of the invention.

Viewed from this aspect, the invention provides an aqueous assay reagent containing a chaotropic salt, an immuno-precipitation enhancer (e.g. PEG), a primary antibody and a secondary antibody.

The invention will now be illustrated further by reference to the following non-limiting Examples and to the accompanying drawings, in which:

Figure 1 shows a plot of absorption against time for calibration samples under the assay system of Example 8;
Figure 2 shows a plot of absorption against time for calibration samples under the assay system of Example 9;
Figure 3 shows dose response curves for the assays using the polyhaptens of Examples 4 and 5;
Figure 4 shows a comparison of immunoprecipitation signal obtained using BSA-SAH, IgY-SAH and PTG-SAH conjugates under the assay conditions of Example 8;
Figure 5 shows a plot of absorption against time for calibration samples under the assay system of Example 10;and
Figure 6 shows the dose response curve for the assay of Example 10.

EXAMPLE 1

Primary Antibody Production

Balb/c mice were immunised with SAH coupled to bovine serum albumin. After the fourth injection, splenocytes from the mice were fused with myeloma cells as described by Gaffre et al. "Preparation of Monoclonal Antibodies: Strategies and Procedures", Meth. Enzymology pages 31-46 (1987). The hybrid cell supernatants were screened for binding to SAH and BSA and those binding to SAH but not BSA were selected for cloning. After stabilising the clone, the cells were grown *in vitro* in hollow fibre systems and the antibodies were purified from the

supernatant using Protein A-sepharose column chromatography (see Ey et al. Immunochem. 15: 429-436 (1978)).

EXAMPLE 2

Secondary Antibody Production

Commercially available polyclonal anti-mouse IgG antibodies may be used. These are produced by injecting mouse IgG into another species (e.g. chicken, goat, rabbit, etc), harvesting antisera and purifying the harvested antisera by immunoaffinity chromatography using antigens coupled to agarose gels.

In the following Examples the secondary antibody is LAJD-112 from La Jolla Diagnostics Inc, California.

(The secondary antibody may alternatively be a monoclonal antibody against the polyhapten:primary antibody complex. In this event it may be produced analogously to the antibody of Example 1 using in place of the SAH-bovine serum albumin conjugate the polyhapten:primary antibody complex or a fragment thereof including the binding site between the polyhapten and the primary antibody).

EXAMPLE 3

Polyhapten production

(A) 150 μL succinic anhydride (7.5 mg/mL in N,N-dimethylformamide) was added dropwise to 5 mL of porcine thyroglobulin (PTG) (5 mg/mL in 10 mM phosphate buffer, pH 7.4). The mixture was allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The product of step (A) was dialysed against 2L of 2-morpholinoethanesulphonic acid (MES) buffer, pH 5.5.

(C) 10 mL of the modified PTG from step (B) (approx. 5 mg/mL in the MES buffer) was mixed with 6.8 mL SAH solution (17 mg in 6.8 mL MES buffer) and 0.7 mL sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL MES buffer).

(D) With constant stirring, 7.6 mg (1-ethyl-3(3-dimethylaminopropyl)carbodiimide.HCl (EDC) was added and the mixture was allowed to react at 20-25°C for 3-5 hours.

(E) The product of step (D) was dialysed against 5L of 10 mM phosphate buffer, pH 7.4. The PTG-polySAH product is used as a polyhapten.

Bovine serum albumin-SAH polyhapten (BSA-SA-SAH) is produced by succinylation followed by carbodiimide coupling using water soluble or insoluble carbodiimides, e.g. EDC/EDAC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-p-toluene sulphonate, or dicyclohexylcarbodiimide, IgG-SAH and IgY (chicken immunoglobulin)-SAH polyhaptens are produced analogously. Dextran-SAH polyhapten is prepared by reaction of amino-dextran with succinic anhydride followed by SAH coupling using a carbodiimide. Alginic acid-SAH is prepared by carbodiimide coupling analogously to steps (D) and (E) in Example 3.

EXAMPLE 3

Polyhapten production

(A) 120 µl succinic anhydride (7.5 mg/mL in N,N-dimethylformamide (DMF)) is added dropwise to 1 ml of poly-L-glutamic acid (10 mg/mL dissolved in 10 mM

phosphate buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The resulting modified (N-terminal blocked) polyglutamic acid is dialysed against 1L of MES buffer, pH 5.5.

(C) 1.6 ml of the dialysed product (approx. 7 mg/ml in MES buffer) is mixed with 0.8 ml SAH solution (1.9 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.8 ml sulpho-N-hydroxysuccinimide solution (0.5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 22 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (s-poly-Glu-SAH) is dialysed against 3L of 10 mM phosphate buffer, pH 7.4.

EXAMPLE 5

Polyhapten production

(A) 150 µl succinic anhydride (7.5 mg/ml in DMF) is added dropwise to 2 ml of casein (5 mg/ml in 10 mM phosphate buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The resulting modified casein is dialysed against 1L of MES buffer, pH 5.5.

(C) 1 ml of the modified casein (approx. 4 mg/ml in 100 mM MES buffer, pH 5.5) is mixed with 0.5 ml SAH solution (2.5 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.07 ml sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 1.2 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (s-casein-SAH) is dialysed against 3L of 10 mM phosphate buffer, pH 7.4.

EXAMPLE 6

Polyhapten preparation

(A) 150 µl succinic anhydride (100 µmol/ml in DMF) is added dropwise to 2 ml of IgY (chicken antibody, 46 mg/ml in 10 mM PBS-buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25 °C with constant stirring.

(B) The resulting modified IgY is dialysed against 10mM MES-buffer, pH 5.5.

(C) 1 ml of the modified IgY (approx. 4 mg/ml in 100 mM MES buffer, pH 5.5) is mixed with 0.5 ml SAH solution (2.5 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.07 ml sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 1.2 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (sIgY-SAH) is dialysed against 3 L of 10mM PBS-buffer.

EXAMPLE 7

Polyhapten preparation

(A) 1 mL of 0.1M $NaIO_4$ is added dropwise to 1 ml mannan (5 mg/ml in water). The mixture is allowed to react for

30 minutes at 20-25°C with constant stirring.

(B) The modified mannan is dialysed against 1L of 10 mM carbonate-buffer, pH 9.5.

(C) 1 mL of the modified mannan (approx. 4 mg/ml in 10 mM carbonate buffer, pH 9.5) is mixed with 1 ml SAH solution (1.9 mg/ml in 10 mM carbonate buffer, pH 9.5). The temperature is maintained at 2-10°C and the reaction is allowed to proceed for 10 hours.

(D) 20 µl 0.1M aqueous $NaBH_4$ is added and the mixture is incubated at 20-25°C for 3 hours.

(E) The mannan-SAH conjugate is dialysed against 1.5L of 10 mM phosphate buffer, pH 7.4.

Figure 4 of the accompanying drawings shows a comparison of the immunoprecipitation signal detected using sBSA-, sIgY-, and sPTG-SAH conjugates as the polyhapten. As can be seen, the precipitation properties of the different conjugates do vary.

The immunoprecipitation ability of the polyhaptens of the Examples is PTG-poly-SAH > sIgY-SAH > mannan-SAH > BSA-SA-SAH = s-poly-Glu-SA > s-Casein-SAH > alginic acid-SAH.

EXAMPLE 8

Two Reagent Assay

Reagent 1

10 mM phosphate buffer*, 0.15M NaCl, pH 7.4	4800 µL
30% w/v PEG 6000	1200 µL
10% Triton X-100 (aqueous)	350 µL

2 mM Adenosine (in phosphate buffer*)	15 µL
50 mM TCEP (aq)	140 µL
PTG-SAH polyhapten (2mg/mL)	400 µL

Reagent 2

10 mM phosphate buffer*	730 µL
30% w/v PEG 6000	270 µL
Primary antibody (3 mg/mL)	400 µL
SAH hydrolase (≈ 350 U/mL)	600 µL

Assay Protocol

10 µL sample (e.g. human plasma) is mixed with 180 µL of Reagent 1 and incubated for 0.25 to 5 minutes at 37°C. 50 µL of Reagent 2 is added, the mixture is incubated for 3 to 10 minutes at 37°C and the precipitation signal is determined by absorption measurements at 340-450 nm, preferably 340 nm.

The assay is calibrated with L-homocyst(e)ine standards.

The total assay time can be as little as 3 minutes. Figure 1 of the accompanying drawings shows a plot of absorption vs time for calibration samples. The jump at measuring point 12 marks the addition of Reagent 2. The assay time may be shortened by careful timing of Reagent addition and absorption measurement and using the difference between measurement at two times after addition of Reagent 2 (e.g. measuring points 15 and 21 in Figure 1). This may be done even though the signal strength may not have reached it maximum at the later point.

EXAMPLE 9

Two Reagent Assay

Reagent 1

As Example 8

Reagent 2

10 mM phosphate buffer*	625 µL
30% w/v PEG 600	270 µL
Primary antibody (3 mg/mL)	400 µL
SAH hydrolase (≈ 350 U/mL)	600 µL
5M NaSCN (aq.)	70 µL
Secondary antibody (rabbit-anti-mouse) (2 mg/mL)	35 µL

Assay Protocol

As for Example 8

Fig. 2 of the accompanying drawings shows a plot of absorption vs. time for a series of calibration samples.

The dose response curve for the assays using the polyhaptens of Examples 4 and 5 are shown in Figure 3 of the accompanying drawings. These are obtained by plotting the absorption difference (dA 340 nm) between the measurements at measuring points 15 and 21 (ie. 7.5 and 10.5 minutes).

EXAMPLE 10

Two Reagent Assay

Reagent 1

10 mM Bis-Tris, pH 6.4	190 µL
Triton X-100 (aqueous)	50 µL
0.5 mM Adenosine (aqueous)	128 µL

5 mM TCEP in Bis-Tris pH 6.4	60 µL
water	233 µL
1M NaSCN (aqueous)	50 µL
1.5 M NaCl (aqueous)	79 µL
PTG-SAH polyhapten (≈ 2mg/mL)	210 µL

Reagent 2

10% PEG (aqueous)	900 µL
100 mM phosphate-buffer, pH 7.4	210 µL
water	140 µL
1.5 M NaCl	220 µL
SAH hydrolase (≈ 350 U/mL)	230 µL
Primary antibody (3 mg/mL)	300 µL

Assay Protocol

10 µL sample (e.g. human plasma) is mixed with 50 µL of Reagent 1 and incubated for 5 minutes at 37°C. 200 µL of Reagent 2 is added, the mixture is incubated for 5 minutes at 37°C and the precipitation signal is determined by absorption measurements at 340-450 nm, preferably 340 nm.

The assay is calibrated with L-homocyst(e)ine standards.

Figure 5 of the accompanying drawings shows a plot of absorption vs time for calibration samples. Dose response curves can be derived from the signal difference between points over the final 5 minutes of the reaction. The dose response curve shown in Figure 6 is derived by plotting the absorption difference between points 13 and 18 as a function of calibrator concentration.